TRANSLATIONAL REVIEW

The Lymphangioleiomyomatosis Lung Cell and Its Human Cell Models

Wendy K. Steagall[1], Gustavo Pacheco-Rodriguez[1], Thomas N. Darling[2], Olga Torre[3], Sergio Harari[3], and Joel Moss[1]

[1]Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; [2]Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, Maryland; and [3]Unità Operativa di Pneumologia e Terapia Semi-Intensiva Respiratoria, Servizio di Fisiopatologia Respiratoria ed Emodinamica Polmonare, Ospedale San Giuseppe, MultiMedica Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy

Abstract

Lymphangioleiomyomatosis (LAM) is a multisystem disease of women, affecting lungs, kidneys, and lymphatics. It is caused by the proliferation of abnormal smooth muscle–like LAM cells, with mutations and loss of heterozygosity in the *TSC1* or, more frequently, *TSC2* genes. Isolated pulmonary LAM cells have been difficult to maintain in culture, and most studies of LAM lung cells involve mixtures of *TSC2* wild-type and *TSC2*-null cells. A clonal population of LAM lung cells has not been established, making analysis of the cells challenging. Cell lines have been established from angiomyolipomas, a common manifestation of LAM, and from tumors from patients with TSC. Circulating LAM cells have also been isolated from blood and other body fluids. LAM cells may also be identified in clusters apparently derived from lymphatic vessels. Genetics, patterns of antigen expression, and signaling pathways have been studied in LAM lung tissue and in LAM cell models, although rarely all in the same study. We show here that LAM cells manifest differences in these characteristics, depending on the source investigated, suggesting further studies.

Keywords: lymphangioleiomyomatosis; tuberous sclerosis; loss of heterozygosity; *TSC2*

Lymphangioleiomyomatosis (LAM) is a multisystem disease occurring predominantly in women, involving lungs, the lymphatic system, and kidneys (Figure 1). LAM can occur sporadically or in conjunction with tuberous sclerosis complex (TSC), an autosomal dominant disorder characterized by hamartomatous growths in the central nervous system, skin, heart, liver, and eyes. Diagnosis is by biopsy showing positive reactivity with monoclonal antibody HMB45 (1), which recognizes the gp100 melanocytic protein, or by high-resolution computed tomography scan revealing characteristic pulmonary cysts plus the presence of TSC, angiomyolipoma (AML), lymphangioleiomyoma (LLM, lymphatic masses due to dilation of lymph vessels) (2), or high serum concentrations of vascular endothelial growth factor (VEGF)-D (3).

The LAM cell is most commonly defined as an abnormal smooth muscle–like cell with inactivating mutations in *TSC1* (chromosome 9q34; encoding hamartin) or, predominantly, *TSC2* (16p13; tuberin). In addition, several molecular markers are beginning to emerge as characteristic LAM cell antigens (Table 1). Hamartin and tuberin negatively regulate the mechanistic target of rapamycin (mTOR), such that deficiency/dysfunction of hamartin or tuberin leads to constitutive activation of mTOR and uncontrolled cell growth and proliferation, usually quantified by an increase in phospho-S6 kinase (4, 5). mTOR inhibitors, such as rapamycin (sirolimus), are the only proven therapy for LAM, but although rapamycin slows LAM cell proliferation, it does not cause cell death (6–8). The origin of the LAM cell is unknown. Here, we examine what is known about the LAM lung cell and contrast/compare that to knowledge from models of human cells used to study LAM. This review focuses on the human LAM cell and excludes the valuable animal models (reviewed in Reference 9).

LAM Cell Genetics

LAM cells are usually characterized by *TSC2*-inactivating mutations or loss of heterozygosity (LOH) of specific microsatellite markers on chromosome 16 (4, 10, 11). Due to the prevalence of AMLs in patients with sporadic LAM (12), *TSC2* LOH (10) and somatic *TSC2* mutations (4) were first identified in DNA isolated from renal AMLs. Identical mutations were found in cells microdissected from the LAM lung as in the tissue from the corresponding AML (4), and *TSC2* LOH was also identified, supporting Knudson's "two-hit" tumor suppressor gene model (13) (Figure 2). The AML and pulmonary LAM cells were concordant for LOH at each microsatellite marker, thus suggesting a common genetic origin for AML and pulmonary LAM (4).

Because sporadic LAM results from somatic, and not germline, mutations (Figure 2), it is often difficult to isolate a

(Received in original form November 15, 2017; accepted in final form February 5, 2018)

Correspondence and requests for reprints should be addressed to Joel Moss, M.D., Ph.D., Room 6D05, Building 10, MSC 1590, National Institutes of Health, Bethesda, MD 20892-1590. E-mail: mossj@nhlbi.nih.gov.

Am J Respir Cell Mol Biol Vol 58, Iss 6, pp 678–683, Jun 2018

Originally Published in Press as DOI: 10.1165/rcmb.2017-0403TR on February 6, 2018
Internet address: www.atsjournals.org

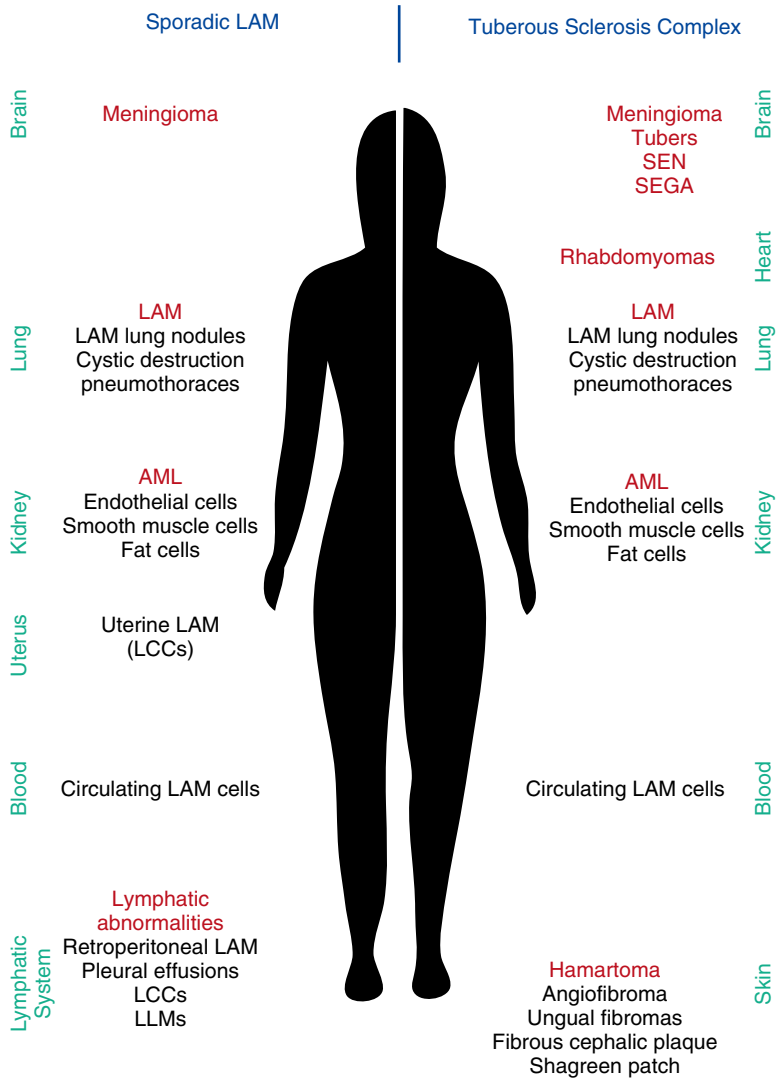

Figure 1. Comparison of the manifestations of sporadic lymphangioleiomyomatosis (LAM) versus TSC. Patients with sporadic LAM present with lung nodules and cystic lung destruction, and may have involvement of the kidneys (angiomyolipomas [AMLs]), uterus, lymphatics (pleural effusions, LLMs), brain (meningioma), and blood (circulating LAM cells). Patients with TSC may present with brain involvement (tubers, subependymal nodules [SEN], subependymal giant cell astrocytomas [SEGA]), heart (rhabdomyomas), lung (LAM lung nodules and cysts), kidney (AML), skin (hamartomas), and blood (circulating LAM cells). LCCs = lymphangioleiomyomatosis cell cluster; LLMs = lymphangioleiomyoma; TSC = tuberous sclerosis complex.

sufficiently pure population of "tumor" cells to successfully sequence or perform LOH analysis or biochemical assays without the data from the wild-type cells overwhelming the analysis. Badri and colleagues (14) used next-generation sequencing to look for *TSC2* mutations in DNA isolated from microdissected LAM lung nodules from 10 patients with sporadic LAM. *TSC2* mutations were found in eight samples, with variant frequencies ranging from 4% to 60%, despite the enrichment of sample by microdissection. Four of the eight had a detectable second-hit inactivation of *TSC2* (three with LOH, one with a second mutation), whereas four had such low mutation frequencies for the first *TSC2* mutation that it was difficult to experimentally detect LOH. Interestingly, two of the samples had neither a *TSC2* nor *TSC1* mutation, and had no evidence of mTOR activation, as determined by the presence of phospho-S6 kinase. These studies underline the importance of cell enrichment for genetic analysis and suggest that alternative genetic changes may be present in LAM.

LAM Cells in Tissues

LAM lung nodules are composed of more proliferative spindle-shaped cells and less proliferative, differentiated epithelioid cells, both of which express α-smooth muscle actin (1, 5, 15). The epithelioid cells are more likely to react with HMB45 (15–95% of cells are reactive in lung biopsy or transplant tissue) (16). LAM nodules also contain type II pneumocytes, lymphatic endothelial cells, and mast cells (17–19). Wild-type fibroblast-like cells have been identified in LAM lung nodules that may provide the proper environment for LAM cell growth (20). Reactivity to antibodies to high-mobility group A2 was detected in all lung tissue samples from 21 patients with LAM, and it is suggested that misexpression of this gene activates a tumorigenic pathway, leading to a benign mesenchymal tumor (21).

Lung biopsy and transplant tissue from patients with sporadic LAM revealed strong positive reactivity with an anti-podoplanin antibody in LAM cells and lymphatic endothelial cells (16), which line enlarged lymphatic capillaries infiltrating the LAM lung nodules (18). Antibodies to lymphatic markers (e.g., vascular endothelial growth factor receptor [VEGFR]-3) show more reactivity in tissue from late-stage LAM (lung explant) than early-stage LAM (lung biopsy) (16). By immunohistopathology, LAM lung nodules are reactive to antibodies against diverse molecules, including hormone and chemokine receptors (summarized in Table 1). The different protein markers on LAM cells from several sources suggest a potential process of cell differentiation within specific microenvironments, and may also suggest that the gene expression of these markers is modified by the cell's microenvironment (e.g., soluble factors, cell–cell interaction).

AMLs are composed of smooth muscle, fat, and vascular components. Both isolated smooth muscle cells and fat cells exhibit *TSC2*LOH. Five different vessel types were identified in AMLs from four patients with sporadic LAM: cellular, collagenous, hemangiopericytic, glomeruloid, and aneurysmatic (22). *TSC2*LOH was detected in all but the collagenous type.

LAM cells infiltrate the walls of lymphatics, causing obstruction of the

Table 1. Characteristics of Human Lymphangioleiomyomatosis Cells (besides gp100 and Smooth Muscle Actin) from Various Tissues

	Lungs	Kidneys	Uterus	Skin	Lymphatic System	Circulating Cells
Genetics	Mutation, LOH (4, 10)	Mutation, LOH, promoter methylation (4, 10, 37)		Mutation (40)	LOH in lymph nodes (10)	LOH (41)
Markers	ER (53–55), PR (53, 56), CD44 (57), CD44v6 (57), PRLr (58), EPOR (44), syndecan-1/2 (27), EGFR (55), CD90 (59), chemokine receptors (43, 60), CD63 (61), β-catenin (62), E-cadherin (55), podoplanin (55), TRAIL (27), RANKL (27), galectin-3 (63), MMPs (54, 64), RAS (19), IGF system (65), COX-2 (31), HMGA2 (21), VEGFR-3 (66), TRP-1 (67), TRP-2 (67), MART-1 (67), CA IX (33), tyrosine kinase Syk (75)	Chemokine receptors (60), EGFR (36, 37), CD44v6 (36, 37), galectin-3 (63), COX-2 (31)	VEGFR-3 (23, 68)	CD9 (41), galectin-3 (63)	VEGFR-3 (18, 23, 68), podoplanin (18, 23), ER (69), PR (69)	CD235a (blood), CD9 and CD44v6 (urine, chyle, BALF) (41)
mTOR	Activated (70, 71)	Activated (72)		Activated (47)	Activated (73)	
Growth factors/ chemokines	PRL (58), OPG (27), EGF (55), CCL2 and other chemokines (43), VEGF-C (23, 66), VEGF-D (25, 66), estradiol (54), PDGF (71)	EGF (36, 37), CXCL12 (60), CX3CL1 (60), CCL11 (60), CCL24 (60), CCL28 (60), estradiol (29)	VEGF-C (23), VEGF-D (25)	CCL2 (42), epiregulin (74)	VEGF-C (23, 66), VEGF-D (25, 66)	
Motility	OPG (27), CCL2 (43)					

Definition of abbreviations: BALF = BAL fluid; CA IX = carbonic anhydrase IX; COX-2 = cyclooxygenase-2; EGF = epithelial growth factor; EGFR = EGF receptor; EPOR = erythropoietin receptor; ER = estrogen receptor; HMGA2 = high-mobility group A2; IGF = insulin-like growth factor; LOH = loss of heterozygosity; MART-1 = melanoma antigen recognized by T cells; MMPs = matrix metalloproteinases; mTOR = mechanistic target of rapamycin; OPG = osteoprotegerin; PDGF = platelet-derived growth factor; PR = progesterone receptor; PRL = prolactin; PRLr = PRL receptor; RANKL = receptor activator of NF-κB ligand; RAS = renin–angiotensin system; TRAIL = TNF-related apoptosis–inducing ligand; TRP = tyrosinase-related protein; VEGF-D = vascular endothelial growth factor D; VEGFR-3 = VEGF receptor 3.

lymph nodes or lymphatic vessels, leading to lymphangioleiomyoma, adenopathy, and/or chylous effusions. LAM cell clusters, nests of HMB45-positive LAM cells enveloped by lymphatic endothelial cells that express VEGFR-3 and podoplanin, have been identified in chylous fluid, the uterus, and within the lumen of lymphatic vessels that infiltrate the LAM nodule (23, 24). VEGF-D and VEGFR-3 were also detected in LAM lung nodules (18). Because serum VEGF-D levels correlate with the degree of lymphatic involvement and disease severity (25, 26), it has been proposed that VEGF-D, acting through VEGFR-3, may facilitate the growth of lymphatic vessels in the LAM nodule and be involved in metastasis.

Human LAM Cell Models

Pulmonary

Cultures of cells derived from LAM lung, after biopsy or transplant, grow as a mixture of *TSC2* wild-type and *TSC2*-null cells, with increased activation of mTOR (5, 27). A homogeneous clonal population of *TSC2*-null pulmonary cells has not been established. An attempt to reprogram LAM lung cells derived from transplant resulted in induced pluripotent stem cells (iPSC) lines that exhibited normal *TSC2* and *TSC1* expression, suggesting that *TSC2* deficiency inhibited production of iPSC lines (28). LAM lung cells, as defined by *TSC2* mutation and/or LOH, do not seem to grow as a clonal population in cell culture; these cells are only detected in the presence of *TSC2* wild-type cells after enrichment.

Kidney

AML cells have been used as surrogates to understand LAM lung cells. The 621–101 cells were derived from an AML and have a *TSC2* mutation (G1832A or R611Q) and *TSC2*LOH at chromosome 16 marker D16S291 (29). These cultures have been used to elucidate the role of estrogens (29, 30), prostaglandins (31), and autophagy (32). 621–101 AML cells have also been used in co-cultures with LAM-associated fibroblasts (WT fibroblast-like cells from LAM lung nodules) to demonstrate the need for both cell types to provide the proper environment for cathepsin K activation (33). The 621–101 line was immortalized by transfection with HPV E6/E7 and telomerase (34). Upon transfection of this cell line with *TSC2*, the cell morphology changed from spindle-shaped ($TSC2^{-}$) to cuboidal ($TSC2^{+}$). These cells also took on a lymphatic endothelial phenotype with expression of markers such as VEGFR-3 and podoplanin, suggesting a lymphatic origin for the LAM cell (35).

Lesma and colleagues isolated cells from AMLs from a female (36) and male patient with TSC (37). Although both cell lines had germline mutations in *TSC2*, one line had *TSC2*LOH, whereas the other had promoter methylation, and thereby epigenetic silencing, of *TSC2*. The cell lines expressed CD44v6, and both required epidermal growth factor for growth.

Skin

Because of the challenges obtaining a homogeneous clonal population of *TSC2*-null pulmonary cells, we have used other

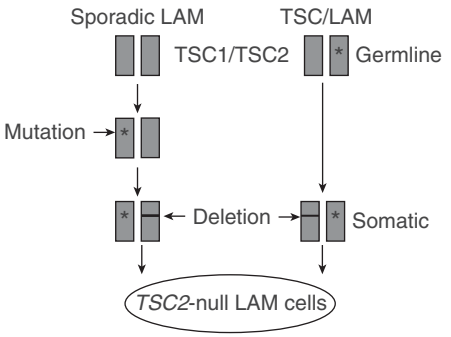

Figure 2. LAM may be sporadic or occur in association with TSC. In sporadic LAM, germline *TSC1/TSC2* are intact, but mutation of one allele of *TSC1* or *TSC2*, followed by deletion of a region of the other allele near *TSC1* or *TSC2* (causing loss of heterozygosity) in somatic cells results in *TSC2*-null LAM cells. In TSC/LAM, germline *TSC1* or *TSC2* is mutated, resulting in somatic cells with mutations and eventual deletion of a region of the chromosome in the vicinity of *TSC1* or *TSC2*. The asterisk indicates a mutation, and the dash indicates a deletion.

tumors with loss of *TSC1* or *TSC2*, especially skin tumors, to gain insights that have shown relevance to LAM. Patients with TSC are predisposed to developing tumors in multiple organs (Figure 1) due to germline mutation in *TSC1* or *TSC2* (38) (Figure 2). We found that TSC skin tumors (39) contain fibroblast-like cells with two inactivating mutations in *TSC1* or *TSC2*, and that primary cells from some TSC skin tumors are greatly enriched for two-hit cells (40). Studies using these TSC skin tumor cells have advanced LAM research. The observation of increased CD9 expression in TSC skin tumor cells led to successful use of this marker in the enrichment of LAM cells from BAL fluid (BALF) and urine (41). TSC skin tumor cells overexpressed CCL2 (42), and this chemokine was also implicated in LAM pathogenesis (43). TSC skin tumor cells were used to show that erythropoietin, a factor that may increase disease progression in LAM, stimulates the proliferation of $TSC2^{-/-}$ cells more than $TSC2^{+/-}$ cells (44). Cells grown from TSC skin tumors and normal-appearing skin were reprogrammed into $TSC2^{+/-}$ iPSC lines that recapitulate features of LAM (28).

Skin tumor cells have also been used to create a xenograft model for TSC. The incorporation of *TSC2*-null cells into skin xenografts in mice causes histological changes that mimic those observed in native tumors (e.g., increased blood vessels, proliferation of the overlying epidermis, recruitment of mononuclear phagocytes) (45). Treatment with sirolimus reverses these abnormalities in xenografts (45) and improves patient skin lesions when given orally (46) or topically (47). It is notable that these improvements were observed despite the continued presence of *TSC2*-null cells in treated xenografts (45) and patients (46).

Circulating Cells

LAM cells, as defined by *TSC2*LOH, have been isolated from blood, urine, expectorated chyle, pleural and abdominal chylous fluids, and BALF (41, 48–50). As with tissue, circulating LAM cells need to be enriched to be studied genetically. Density gradient centrifugation of blood is used to enrich tumor cells, followed by FACS with CD45 and CD235a (41, 48). Circulating LAM cells are found independent of disease stage (41, 48). Cells with *TSC2*LOH were found in urine, mostly in patients with AMLs, and in chylous fluid (41), after FACS with antibodies to CD44v6 and CD9, prometastatic molecules that may enable cell mobilization and anchorage to sites of metastasis (41).

LAM cells isolated from different body fluids are phenotypically different: LAM cells from urine and BALF cannot be isolated using anti-CD45 and anti-CD235a antibodies, and those from blood cannot be isolated with anti-CD44v6 and anti-CD44 or anti-CD44v6 and anti-CD9 antibodies (41). In most patients with sporadic LAM, LAM cells isolated from different body fluids of the same patient showed identical *TSC2*LOH patterns for specific microsatellites, consistent with a common genetic origin. However, almost 26% of patients had different patterns of *TSC2*LOH in blood subpopulations (51), suggesting that a patient may have different clones of LAM cells. Different patterns of *TSC2*LOH were also detected in cells from blood versus urine (51). Cells with *TSC2*LOH were isolated from blood of patients with LAM after bilateral lung transplantation, suggesting that circulating LAM cells may originate from somewhere other than the lung (51). Treatment with rapamycin significantly decreased, in a time-dependent manner, the ability to detect cells with *TSC2*LOH in blood and urine (49).

The specificity of *TSC2*LOH in circulating cells as a marker for LAM is not definitive, although it may be a useful marker when coupled with clinical data. In a recent study analyzing samples from patients with different lung diseases (52), cells from blood and urine were isolated and analyzed for *TSC2*LOH. *TSC2*LOH was found in a patient with sarcoidosis in the $CD9^{-}CD44v6^{-}$ cell population from urine and in a case of pulmonary Langerhans cell histiocytosis in unsorted blood cells. Interestingly, one patient with pulmonary Langerhans cell histiocytosis showed *TSC2*LOH in cells expressing CD1a, a marker of Langerhans cells. Cells with *TSC*LOH have been found in patients with cancers, including lung cancer, supporting the hypothesis that *TSC*LOH could be a common event in different cancerous processes.

Conclusions

The phenotype of a LAM cell differs according to its source. The biomarkers that are constant across different types of human LAM cells have not been determined rigorously (Table 1). LAM cells may retain the potential to differentiate (e.g., the different kinds of LAM cells in an AML) or may retain stem cell characteristics. Mutations in *TSC2* also define a LAM cell, although other genetic/biochemical changes cannot be ruled out. The presence of *TSC2*LOH may be diagnostic only with the presence of other clinical factors or cell surface markers. The origin of the LAM cell remains elusive. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Matsumoto Y, Horiba K, Usuki J, Chu SC, Ferrans VJ, Moss J. Markers of cell proliferation and expression of melanosomal antigen in lymphangioleiomyomatosis. *Am J Respir Cell Mol Biol* 1999;21: 327–336.
2. Avila NA, Bechtle J, Dwyer AJ, Ferrans VJ, Moss J. Lymphangioleiomyomatosis: CT of diurnal variation of lymphangioleiomyomas. *Radiology* 2001;221:415–421.
3. McCormack FX, Gupta N, Finlay GR, Young LR, Taveira-DaSilva AM, Glasgow CG, *et al*.; ATS/JRS Committee on Lymphangioleiomyomatosis. Official American Thoracic Society/Japanese Respiratory Society clinical practice guidelines: lymphangioleiomyomatosis diagnosis and management. *Am J Respir Crit Care Med* 2016;194:748–761.
4. Carsillo T, Astrinidis A, Henske EP. Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 2000;97: 6085–6090.
5. Krymskaya VP. Smooth muscle–like cells in pulmonary lymphangioleiomyomatosis. *Proc Am Thorac Soc* 2008;5:119–126.
6. McCormack FX, Inoue Y, Moss J, Singer LG, Strange C, Nakata K, *et al*.; National Institutes of Health Rare Lung Diseases Consortium; MILES Trial Group. Efficacy and safety of sirolimus in lymphangioleiomyomatosis. *N Engl J Med* 2011;364:1595–1606.
7. Krymskaya VP, McCormack FX. Lymphangioleiomyomatosis: a monogenic model of malignancy. *Annu Rev Med* 2017;68:69–83.
8. El-Chemaly S, Taveira-Dasilva A, Goldberg HJ, Peters E, Haughey M, Bienfang D, *et al*. Sirolimus and autophagy inhibition in lymphangioleiomyomatosis: results of a phase I clinical trial. *Chest* 2017;151:1302–1310.
9. Kwiatkowski DJ. Animal models of lymphangioleiomyomatosis (LAM) and tuberous sclerosis complex (TSC). *Lymphat Res Biol* 2010;8: 51–57.
10. Smolarek TA, Wessner LL, McCormack FX, Mylet JC, Menon AG, Henske EP. Evidence that lymphangiomyomatosis is caused by TSC2 mutations: chromosome 16p13 loss of heterozygosity in angiomyolipomas and lymph nodes from women with lymphangiomyomatosis. *Am J Hum Genet* 1998;62:810–815.
11. Yu J, Astrinidis A, Henske EP. Chromosome 16 loss of heterozygosity in tuberous sclerosis and sporadic lymphangiomyomatosis. *Am J Respir Crit Care Med* 2001;164:1537–1540.
12. Taveira-DaSilva AM, Pacheco-Rodriguez G, Moss J. The natural history of lymphangioleiomyomatosis: markers of severity, rate of progression and prognosis. *Lymphat Res Biol* 2010;8:9–19.
13. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 1971;68:820–823.
14. Badri KR, Gao L, Hyjek E, Schuger N, Schuger L, Qin W, *et al*. Exonic mutations of TSC2/TSC1 are common but not seen in all sporadic pulmonary lymphangioleiomyomatosis. *Am J Respir Crit Care Med* 2013;187:663–665.
15. Zhang X, Travis WD. Pulmonary lymphangioleiomyomatosis. *Arch Pathol Lab Med* 2010;134:1823–1828.
16. Davis JM, Hyjek E, Husain AN, Shen L, Jones J, Schuger LA. Lymphatic endothelial differentiation in pulmonary lymphangioleiomyomatosis cells. *J Histochem Cytochem* 2013;61:580–590.
17. Matsui K, K Riemenschneider W, Hilbert SL, Yu ZX, Takeda K, Travis WD, *et al*. Hyperplasia of type II pneumocytes in pulmonary lymphangioleiomyomatosis. *Arch Pathol Lab Med* 2000;124:1642–1648.
18. Glasgow CG, El-Chemaly S, Moss J. Lymphatics in lymphangioleiomyomatosis and idiopathic pulmonary fibrosis. *Eur Respir Rev* 2012;21:196–206.
19. Valencia JC, Pacheco-Rodriguez G, Carmona AK, Xavier J, Bruneval P, Riemenschneider WK, *et al*. Tissue-specific renin-angiotensin system in pulmonary lymphangioleiomyomatosis. *Am J Respir Cell Mol Biol* 2006;35:40–47.
20. Clements D, Dongre A, Krymskaya VP, Johnson SR. Wild type mesenchymal cells contribute to the lung pathology of lymphangioleiomyomatosis. *PLoS One* 2015;10:e0126025.
21. D'Armiento J, Imai K, Schiltz J, Kolesnekova N, Sternberg D, Benson K, *et al*. Identification of the benign mesenchymal tumor gene HMGA2 in lymphangiomyomatosis. *Cancer Res* 2007;67:1902–1909.
22. Karbowniczek M, Yu J, Henske EP. Renal angiomyolipomas from patients with sporadic lymphangiomyomatosis contain both neoplastic and non-neoplastic vascular structures. *Am J Pathol* 2003;162:491–500.
23. Kumasaka T, Seyama K, Mitani K, Sato T, Souma S, Kondo T, *et al*. Lymphangiogenesis in lymphangioleiomyomatosis: its implication in the progression of lymphangioleiomyomatosis. *Am J Surg Pathol* 2004;28:1007–1016.
24. Kumasaka T, Seyama K, Mitani K, Souma S, Kashiwagi S, Hebisawa A, *et al*. Lymphangiogenesis-mediated shedding of LAM cell clusters as a mechanism for dissemination in lymphangioleiomyomatosis. *Am J Surg Pathol* 2005;29:1356–1366.
25. Glasgow CG, Avila NA, Lin JP, Stylianou MP, Moss J. Serum vascular endothelial growth factor-D levels in patients with lymphangioleiomyomatosis reflect lymphatic involvement. *Chest* 2009;135:1293–1300.
26. Young LR, Inoue Y, McCormack FX. Diagnostic potential of serum VEGF-D for lymphangioleiomyomatosis. *N Engl J Med* 2008;358:199–200.
27. Steagall WK, Pacheco-Rodriguez G, Glasgow CG, Ikeda Y, Lin JP, Zheng G, *et al*. Osteoprotegerin contributes to the metastatic potential of cells with a dysfunctional TSC2 tumor-suppressor gene. *Am J Pathol* 2013;183:938–950.
28. Julian LM, Delaney SP, Wang Y, Goldberg AA, Doré C, Yockell-Lelièvre J, *et al*. Human pluripotent stem cell–derived TSC2-haploinsufficient smooth muscle cells recapitulate features of lymphangioleiomyomatosis. *Cancer Res* 2017;77:5491–5502.
29. Yu J, Astrinidis A, Howard S, Henske EP. Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L694–L700.
30. Yu J, Henske EP. mTOR activation, lymphangiogenesis, and estrogen-mediated cell survival: the "perfect storm" of pro-metastatic factors in LAM pathogenesis. *Lymphat Res Biol* 2010;8:43–49.
31. Li C, Lee PS, Sun Y, Gu X, Zhang E, Guo Y, *et al*. Estradiol and mTORC2 cooperate to enhance prostaglandin biosynthesis and tumorigenesis in TSC2-deficient LAM cells. *J Exp Med* 2014;211:15–28.
32. Li C, Li N, Liu X, Zhang EY, Sun Y, Masuda K, *et al*. Proapoptotic protein Bim attenuates estrogen-enhanced survival in lymphangioleiomyomatosis. *JCI Insight* 2016;1:e86629.
33. Dongre A, Clements D, Fisher AJ, Johnson SR. Cathepsin K in lymphangioleiomyomatosis: LAM cell–fibroblast interactions enhance protease activity by extracellular acidification. *Am J Pathol* 2017;187:1750–1762.
34. Siroky BJ, Yin H, Babcock JT, Lu L, Hellmann AR, Dixon BP, *et al*. Human TSC-associated renal angiomyolipoma cells are hypersensitive to ER stress. *Am J Physiol Renal Physiol* 2012;303: F831–F844.
35. Yue M, Pacheco G, Cheng T, Li J, Wang Y, Henske EP, *et al*. evidence supporting a lymphatic endothelium origin for angiomyolipoma, a TSC2(−) tumor related to lymphangioleiomyomatosis. *Am J Pathol* 2016;186:1825–1836.
36. Lesma E, Grande V, Carelli S, Brancaccio D, Canevini MP, Alfano RM, *et al*. Isolation and growth of smooth muscle–like cells derived from tuberous sclerosis complex-2 human renal angiomyolipoma: epidermal growth factor is the required growth factor. *Am J Pathol* 2005;167:1093–1103.
37. Lesma E, Sirchia SM, Ancona S, Carelli S, Bosari S, Ghelma F, *et al*. The methylation of the TSC2 promoter underlies the abnormal growth of TSC2 angiomyolipoma-derived smooth muscle cells. *Am J Pathol* 2009;174:2150–2159.
38. Northrup H, Krueger DA; International Tuberous Sclerosis Complex Consensus Group. Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. *Pediatr Neurol* 2013;49:243–254.
39. Teng JM, Cowen EW, Wataya-Kaneda M, Gosnell ES, Witman PM, Hebert AA, *et al*. Dermatologic and dental aspects of the 2012 International Tuberous Sclerosis Complex Consensus Statements. *JAMA Dermatol* 2014;150:1095–1101.
40. Tyburczy ME, Wang JA, Li S, Thangapazham R, Chekaluk Y, Moss J, *et al*. Sun exposure causes somatic second-hit mutations and angiofibroma development in tuberous sclerosis complex. *Hum Mol Genet* 2014;23:2023–2029.

41. Cai X, Pacheco-Rodriguez G, Fan QY, Haughey M, Samsel L, El-Chemaly S, *et al*. Phenotypic characterization of disseminated cells with TSC2 loss of heterozygosity in patients with lymphangioleiomyomatosis. *Am J Respir Crit Care Med* 2010;182:1410–1418.
42. Li S, Takeuchi F, Wang JA, Fuller C, Pacheco-Rodriguez G, Moss J, *et al*. MCP-1 overexpressed in tuberous sclerosis lesions acts as a paracrine factor for tumor development. *J Exp Med* 2005;202:617–624.
43. Pacheco-Rodriguez G, Kumaki F, Steagall WK, Zhang Y, Ikeda Y, Lin JP, *et al*. Chemokine-enhanced chemotaxis of lymphangioleiomyomatosis cells with mutations in the tumor suppressor TSC2 gene. *J Immunol* 2009;182:1270–1277.
44. Ikeda Y, Taveira-DaSilva AM, Pacheco-Rodriguez G, Steagall WK, El-Chemaly S, Gochuico BR, *et al*. Erythropoietin-driven proliferation of cells with mutations in the tumor suppressor gene TSC2. *Am J Physiol Lung Cell Mol Physiol* 2011;300:L64–L72.
45. Li S, Thangapazham RL, Wang JA, Rajesh S, Kao TC, Sperling L, *et al*. Human TSC2-null fibroblast-like cells induce hair follicle neogenesis and hamartoma morphogenesis. *Nat Commun* 2011;2:235.
46. Nathan N, Wang JA, Li S, Cowen EW, Haughey M, Moss J, *et al*. Improvement of tuberous sclerosis complex (TSC) skin tumors during long-term treatment with oral sirolimus. *J Am Acad Dermatol* 2015;73:802–808.
47. Wataya-Kaneda M, Nakamura A, Tanaka M, Hayashi M, Matsumoto S, Yamamoto K, *et al*. Efficacy and safety of topical sirolimus therapy for facial angiofibromas in the tuberous sclerosis complex : a randomized clinical trial. *JAMA Dermatol* 2017;153:39–48.
48. Crooks DM, Pacheco-Rodriguez G, DeCastro RM, McCoy JP Jr, Wang JA, Kumaki F, *et al*. Molecular and genetic analysis of disseminated neoplastic cells in lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 2004;101:17462–17467.
49. Cai X, Pacheco-Rodriguez G, Haughey M, Samsel L, Xu S, Wu HP, *et al*. Sirolimus decreases circulating lymphangioleiomyomatosis cells in patients with lymphangioleiomyomatosis. *Chest* 2014;145:108–112.
50. Harari S, Elia D, Torre O, Bulgheroni E, Provasi E, Moss J. Sirolimus therapy for patients with lymphangioleiomyomatosis leads to loss of chylous ascites and circulating LAM cells. *Chest* 2016;150:e29–e32.
51. Steagall WK, Zhang L, Cai X, Pacheco-Rodriguez G, Moss J. Genetic heterogeneity of circulating cells from patients with lymphangioleiomyomatosis with and without lung transplantation. *Am J Respir Crit Care Med* 2015;191:854–856.
52. Zhang L, Pacheco-Rodriguez G, Steagall WK, Kato J, Haughey M, Fontana JR, *et al*. Tuberous sclerosis complex 2 loss of heterozygosity in patients with lung disease and cancer. *Am J Respir Crit Care Med* 2015;191:352–355.
53. Matsui K, Takeda K, Yu ZX, Valencia J, Travis WD, Moss J, *et al*. Downregulation of estrogen and progesterone receptors in the abnormal smooth muscle cells in pulmonary lymphangioleiomyomatosis following therapy: an immunohistochemical study. *Am J Respir Crit Care Med* 2000;161:1002–1009.
54. Glassberg MK, Elliot SJ, Fritz J, Catanuto P, Potier M, Donahue R, *et al*. Activation of the estrogen receptor contributes to the progression of pulmonary lymphangioleiomyomatosis via matrix metalloproteinase–induced cell invasiveness. *J Clin Endocrinol Metab* 2008;93:1625–1633.
55. Grzegorek I, Lenze D, Chabowski M, Janczak D, Szolkowska M, Langfort R, *et al*. Immunohistochemical evaluation of pulmonary lymphangioleiomyomatosis. *Anticancer Res* 2015;35:3353–3360.
56. Gao L, Yue MM, Davis J, Hyjek E, Schuger L. In pulmonary lymphangioleiomyomatosis expression of progesterone receptor is frequently higher than that of estrogen receptor. *Virchows Arch* 2014; 464:495–503.
57. Pacheco-Rodriguez G, Steagall WK, Crooks DM, Stevens LA, Hashimoto H, Li S, *et al*. TSC2 loss in lymphangioleiomyomatosis cells correlated with expression of CD44v6, a molecular determinant of metastasis. *Cancer Res* 2007;67:10573–10581.
58. Terasaki Y, Yahiro K, Pacheco-Rodriguez G, Steagall WK, Stylianou MP, Evans JF, *et al*. Effects of prolactin on TSC2-null Eker rat cells and in pulmonary lymphangioleiomyomatosis. *Am J Respir Crit Care Med* 2010;182:531–539.
59. Ando K, Fujino N, Mitani K, Ota C, Okada Y, Kondo T, *et al*. Isolation of individual cellular components from lung tissues of patients with lymphangioleiomyomatosis. *Am J Physiol Lung Cell Mol Physiol* 2016;310:L899–L908.
60. Clements D, Markwick LJ, Puri N, Johnson SR. Role of the CXCR4/CXCL12 axis in lymphangioleiomyomatosis and angiomyolipoma. *J Immunol* 2010;185:1812–1821.
61. Zhe X, Schuger L. Combined smooth muscle and melanocytic differentiation in lymphangioleiomyomatosis. *J Histochem Cytochem* 2004;52:1537–1542.
62. Flavin RJ, Cook J, Fiorentino M, Bailey D, Brown M, Loda MF. β-Catenin is a useful adjunct immunohistochemical marker for the diagnosis of pulmonary lymphangioleiomyomatosis. *Am J Clin Pathol* 2011;135:776–782.
63. Klover PJ, Thangapazham RL, Kato J, Wang JA, Anderson SA, Hoffmann V, *et al*. Tsc2 disruption in mesenchymal progenitors results in tumors with vascular anomalies overexpressing Lgals3. *eLife* 2017;6:pii: e23202.
64. Hayashi T, Fleming MV, Stetler-Stevenson WG, Liotta LA, Moss J, Ferrans VJ, *et al*. Immunohistochemical study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pulmonary lymphangioleiomyomatosis (LAM). *Hum Pathol* 1997;28: 1071–1078.
65. Valencia JC, Matsui K, Bondy C, Zhou J, Rasmussen A, Cullen K, *et al*. Distribution and mRNA expression of insulin-like growth factor system in pulmonary lymphangioleiomyomatosis. *J Investig Med* 2001;49:421–433.
66. Issaka RB, Oommen S, Gupta SK, Liu G, Myers JL, Ryu JH, *et al*. Vascular endothelial growth factors C and D induces proliferation of lymphangioleiomyomatosis cells through autocrine crosstalk with endothelium. *Am J Pathol* 2009;175: 1410–1420.
67. Klarquist J, Barfuss A, Kandala S, Reust MJ, Braun RK, Hu J, *et al*. Melanoma-associated antigen expression in lymphangioleiomyomatosis renders tumor cells susceptible to cytotoxic T cells. *Am J Pathol* 2009;175:2463–2472.
68. Hayashi T, Kumasaka T, Mitani K, Terao Y, Watanabe M, Oide T, *et al*. Prevalence of uterine and adnexal involvement in pulmonary lymphangioleiomyomatosis: a clinicopathologic study of 10 patients. *Am J Surg Pathol* 2011;35:1776–1785.
69. Matsui K, Tatsuguchi A, Valencia J, Yu Zx, Bechtle J, Beasley MB, *et al*. Extrapulmonary lymphangioleiomyomatosis (LAM): clinicopathologic features in 22 cases. *Hum Pathol* 2000;31: 1242–1248.
70. Goncharova EA, Goncharov DA, Eszterhas A, Hunter DS, Glassberg MK, Yeung RS, *et al*. Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation: a role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J Biol Chem* 2002;277:30958–30967.
71. Goncharova EA, Goncharov DA, Spaits M, Noonan DJ, Talovskaya E, Eszterhas A, *et al*. Abnormal growth of smooth muscle–like cells in lymphangioleiomyomatosis: role for tumor suppressor TSC2. *Am J Respir Cell Mol Biol* 2006;34:561–572.
72. Robb VA, Astrinidis A, Henske EP. Frequent [corrected] hyperphosphorylation of ribosomal protein S6 [corrected] in lymphangioleiomyomatosis-associated angiomyolipomas. *Mod Pathol* 2006;19:839–846. [Published erratum appears in *Mod Pathol* 19:889.]
73. Taveira-DaSilva AM, Hathaway O, Stylianou M, Moss J. Changes in lung function and chylous effusions in patients with lymphangioleiomyomatosis treated with sirolimus. *Ann Intern Med* 2011;154:797–805, W-292–W-793.
74. Li S, Takeuchi F, Wang JA, Fan Q, Komurasaki T, Billings EM, *et al*. Mesenchymal–epithelial interactions involving epiregulin in tuberous sclerosis complex hamartomas. *Proc Natl Acad Sci USA* 2008;105: 3539–3544.
75. Cui Y, Steagall WK, Lamattina AM, Pacheco-Rodriguez G, Stylianou M, Kidambi P, *et al*. Aberrant SYK kinase signaling is essential for tumorigenesis induced by TSC2 inactivation. *Cancer Res* 2017;77: 1492–1502.